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Detection of the Latent Form of Epstein-Barr Virus DNA in the Peripheral Blood of Healthy Individuals

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Epstein-Barr virus infects resting B cells in vitro and activates them to continuously proliferating lymphoblasts. Activation is essential for the virus to convert its linear genome to the covalently closed circular episomal form in which it persists in proliferating cells. However, in vivo, Epstein-Barr virus persists in resting B cells. We found that in these cells also the virus is present as an episome, suggesting that the cells must, at some time, have been activated and then returned to a resting state. This is the first direct demonstration, for any herpesvirus, of this form of the viral genome in normal persistently infected tissue. Since no linear viral DNA was detected, we estimate that fewer than 1 in 40 cells replicates the virus in the peripheral blood of healthy donors.

Epstein-Barr virus (EBV), like other herpesviruses, is characterized by the ability to persist for the lifetime of the infected host (11). The resultant lifetime exposure of tissues to the virus and the immortalizing power of EBV are thought to be major contributing factors to the development of EBV-associated malignancies.

Recently, we have obtained evidence that most of the persistently EBV-infected cells in the peripheral blood of healthy donors are resting B cells (11a, 12). The detection of EBV in normal resting B cells presents a new paradigm for EBV persistence since the virus has always been found previously in association with proliferating cells, including B-cell lines (7), the EBV-positive tumors Burkitt's and Hodgkin's lymphomas, immunoblastic lymphomas in the immunosuppressed, and nasopharyngeal carcinoma. The question arises, therefore, as to the mode of persistence of the viral genome in a resting B cell.

EBV can infect a variety of B cells in vitro but preferentially immortalizes normal resting B cells (16). The viral genome is in the linear form when it enters the nucleus and remains this way until the infected cell exists from the resting state (6). Traversal of the first G_1 prior to entry into the cell cycle is essential for the linear genome to circularize into a covalently closed circular episome (6), the form in which the virus generally persists in proliferating cells (13, 15).

One exception occurs when established EBV-negative B-cell tumor lines are infected in vitro. In this case, a high proportion of the derived cell lines fail to form episomes, possibly because they are already in cycle; instead, the viral genome is integrated (5). One characteristic of the cell lines with integrated EBV is that they have unusually low genome copy numbers (less than five). A low genome copy number is also a characteristic of latently infected normal B cells in vivo (12). Therefore, there are several alternative forms in which the virus could persist in vivo, including linear, episomal, or integrated. Furthermore, it is unknown what fraction of the cells, if any, actively replicates the virus.

In the current study, we used PCR and a modified Gardella gel system (4) to directly demonstrate the presence of intact, full-length episomal EBV DNA in the peripheral blood B cells of healthy, persistently infected individuals. Linear forms of the viral genome were not detected.

Resolution of episomal and linear genomes by Gardella gel. The Gardella technique is an in situ lysis gel that allows the detection of linear, episomal, and integrated forms of EBV DNA on the basis of their characteristic migration. The validity of this method has been established previously (4–6). In a large panel of cell lines which either lack or possess episomal DNA, as judged by restriction enzyme analysis and in situ fluorescence hybridization, the presence of the appropriately migrating DNA on a Gardella gel was consistently diagnostic for the presence or absence of episomal DNA (5). Furthermore, the conversion of the linear to the episomal form in newly infected B cells (6) and of the episomal to the linear form by nicking (unpublished observations) can be demonstrated with Gardella gels.

This technique can also be used to distinguish cells that are latently infected and contain episomes from cells that replicate the virus and contain linear forms of the genome. Figure 1 demonstrates the resolution obtainable. Horizontal gels of 0.75% Seakem LE agarose (FMC) were poured. The 5-cm region above the well was removed and replaced with 0.8% agarose containing 2% sodium dodecyl sulfate (SDS) and 1 mg of self-digested pronase (Calbiochem) per ml. The samples were electrophoresed for 2 h at 40 V and 14 to 18 h at 160 V at 4°C. The DNA was detected by Southern blotting exactly as described previously (6). In this experiment, we fractionated DNAs from the tightly latent cell line RAJI (episomal only), from the B95-8 cell line, which is a mixture of latently and lytically infected cells (episomal and linear), and from B95-8 virions (linear only). Linear and episomal DNAs were clearly resolved.

We measured the frequency of infected B cells in the peripheral blood of normal donors and found that it varied between 1 and $50/10^6$ B cells (6a, 12). To analyze these rare cells by Gardella gel, we modified the technique to allow DNA PCR to be performed on sequential gel slices. Figure 2 shows the results obtained from such an analysis of three cell lines, RAJI (episomal form only), B95-8 (episomal and linear forms), and EBV-negative BJAB. The samples were electrophoresed into 0.75% Seaplaque GTG agarose with TBE (8.9 mM Tris base,

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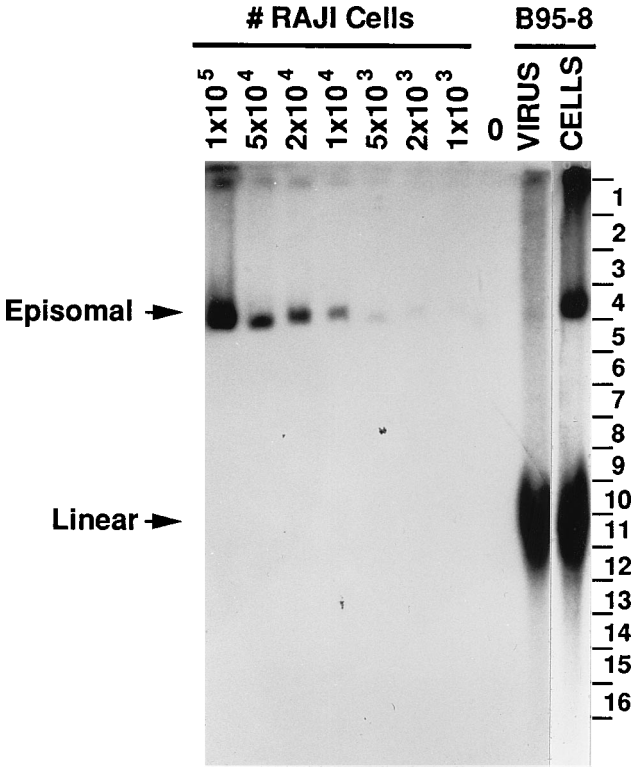

FIG. 1. Resolution of linear and episomal forms of the EBV genome by Gardella gel. Serial dilutions of the tightly latent, EBV-positive cell line RAJI (50 genome copies per cell) (1), supernatant from the B95-8 cell line containing cell-free virus, and B95-8 cells containing a mixture of latently and lytically (about 5 to 10%) infected cells were loaded into the sample wells of a Gardella gel, and the DNA was fractionated and detected by Southern blotting exactly as described previously (6). For the lanes containing RAJI cells, the cell number was increased to 10^6 in each case by addition of EBV-negative BJAB cells. The lane labelled 0 contained only BJAB cells. The numbers on the right represent the approximate locations of the gel slices made in subsequent experiments.

8.9 mM boric acid, 0.8 mM EDTA) as the running buffer. Lanes containing the samples were excised and cut into a series of uniform 0.5-cm slices by using a standardized template. Slices were melted at 65°C for 15 min, and then 5 μl of a 200-μl total volume of molten agarose was used directly for PCR. EBV PCR was performed as previously described (12), with the addition of *Taq* extender (Stratagene). The primers used (forward, 5′ CTTTAGAGGCGAATGGGCGCCA 3′; reverse, 5′ TCCAGGGCCTTCACTTCGGTCT 3′) amplified the sequence between 14068 and 14562 of the *Bam*HI-W repeat of EBV to give a product of 495 bp.

Only covalently closed EBV episomes were detected, at the expected position, in RAJI cells. By comparison, both linear and episomal forms of the virus were found in B95-8 cells. The migration point for the linear form of the viral DNA was also as expected and was confirmed by analyzing supernatant, from the B95-8 cell line, containing cell-free virus. The result obtained with B95-8 cells demonstrates that the linear and episomal forms of the virus were well resolved. Furthermore, to confirm the sensitivity of the technique, 10 B95-8 cells were loaded onto the gel and only 1/40 of the sample from each gel slice was analyzed. Since clear signals were seen in both the linear and episomal regions, we may conclude that the technique can detect EBV DNA from even a fraction of a single latently or lytically infected cell. This was expected, since we have previously demonstrated that our DNA PCR technique can detect a single EBV genome in the presence of up to 10^6 uninfected cells (12).

Detection of episomes in peripheral blood B cells. Having established the resolution and sensitivity of the technique, we proceeded to analyze peripheral blood B cells from healthy donors. B-cell-enriched fractions were prepared by depleting peripheral blood mononuclear cells of T cells (E^- cells; 20 to 50% B cells) as described previously (12). The experiment was performed exactly as for the cell lines in Fig. 2, except that instead of analyzing the melted gel directly, slices were melted at 65°C for 15 min and then digested with β-agarase (FMC) in accordance with the manufacturer's recommendation. The DNA was precipitated by addition of 2.5 M ammonium acetate and 100% ethanol. After washing in 70% ethanol and air drying, the DNA was solubilized in 50 μl of high-pressure liquid chromatography grade H_2O at 4°C overnight and 25 μl was used in a PCR.

The result obtained when peripheral blood B cells from two healthy donors were analyzed by Gardella gel and PCR is shown in Fig. 3. A strong signal was obtained at the expected migration point for intact, covalently closed EBV episomes from both donors. No linear DNA was detected. In all, we detected episomal DNA in five of the five healthy donors tested.

We have previously shown that most of the EBV-carrying B cells in the peripheral blood are in the $CD23^-$ population. To confirm that episomal DNA was also present in this population, we compared E^- (B cells and monocytes) and $CD23^-$ B cells. $CD23^-$ B cells were prepared as described previously (12). As shown in Fig. 4, episomal EBV DNA was readily detected in both populations.

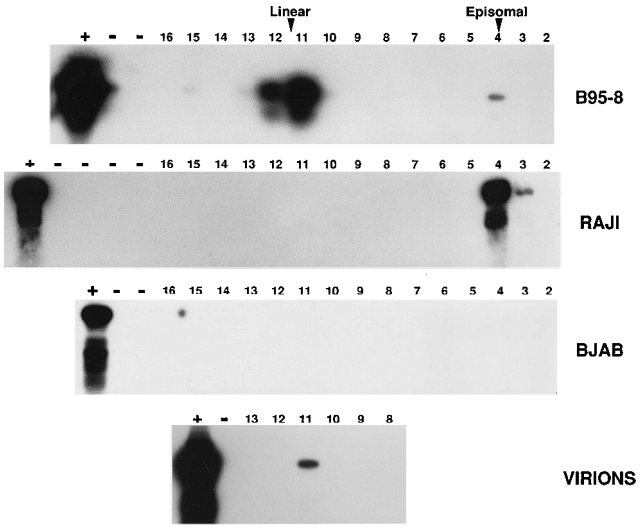

FIG. 2. Detection of episomal and linear forms of the EBV genome in cell lines by PCR analysis of Gardella gels. Cells were fractionated on a Gardella gel. Each lane was then sliced approximately as shown in Fig. 1. DNA was prepared from the slices as described in the text, and 1/40 of each sample was tested for EBV by PCR. The PCR products were fractionated by agarose gel electrophoresis and detected by Southern blotting (12). Lanes: B95-8, 10 B95-8 cells (mixture of latent and lytically infected cells) in 10^6 BJAB (EBV-negative) cells; RAJI, 10^3 RAJI (EBV-positive, tightly latent) cells in 10^6 BJAB cells; BJAB, 10^6 BJAB cells alone; Virions, 1 μl of a 1:250,000 dilution of a cell-free supernatant of virus producer cell line B95-8 (only the linear region of this gel was analyzed); +, positive control for the PCR of 10^3 Namalwa cells (two genome copies per cell). −, PCR analysis of slices from regions of the gel that should not contain EBV DNA, as a negative control. The numbers of the gel slices correspond approximately to the numbering in Fig. 1. Multiple no-DNA and negative-DNA (from the cell line BJAB) samples were analyzed simultaneously as controls and were consistently negative (data not shown).

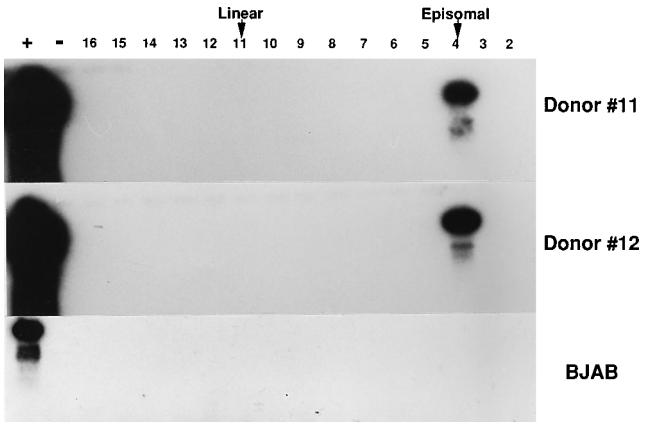

FIG. 3. Detection of episomes and absence of linear genomes in peripheral blood B cells of normal donors. Lymphocyte fractions containing B cells were analyzed for the presence of EBV by Gardella gel followed by DNA PCR of sequential gel slices. As a control, the EBV-negative BJAB cell line was also analyzed. +, positive control for the PCR of 10^3 Namalwa cells (two genome copies per cell). −, PCR analysis of slices from regions of the gel that should not contain EBV DNA, as a negative control. The gel slice numbers correspond approximately to the positions in Fig. 1. In all experiments, several no-DNA and negative-DNA (from the cell line BJAB) samples were analyzed simultaneously as negative controls (data not shown).

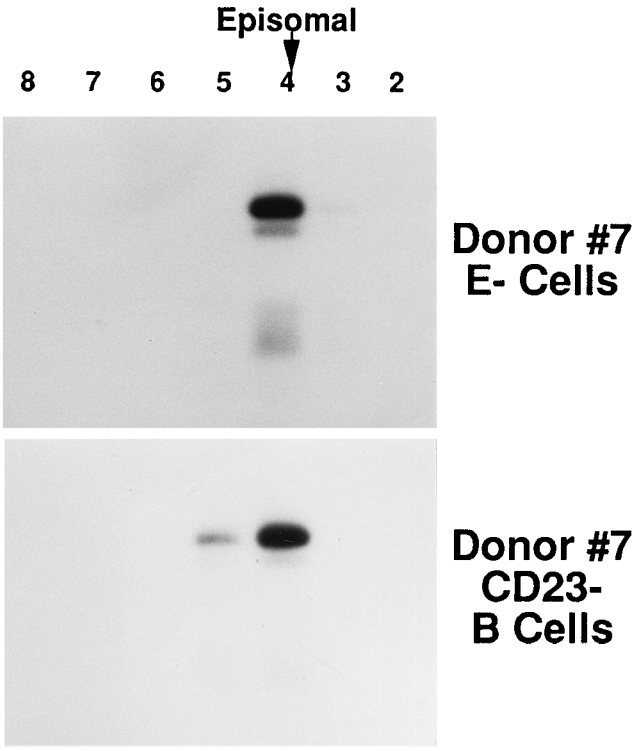

FIG. 4. Detection of episomes in CD23⁻ peripheral blood B cells of normal donors. Lymphocyte fractions containing B cells were analyzed for the presence of the episomal form of EBV by Gardella gel followed by DNA PCR of sequential gel slices. For details of the preparation of E cells (T-cell-depleted peripheral blood mononuclear cells—20 to 50% B cells) and CD23⁻ B cells, see the text. The linear regions of these gels were not analyzed. In all experiments, several no-DNA and negative-DNA (from the cell line BJAB) samples were analyzed simultaneously as negative controls (data not shown).

Detection of linear genomes in short-term cultures of peripheral blood B cells. We did not detect the linear form of the viral genome in the peripheral blood of healthy donors. As described above, control experiments with cell lines demonstrated that we could detect a small fraction of the DNA from a single cell that was replicating the virus in a cell line. We wished to confirm that our approach would also detect such cells in peripheral blood if they were present. Therefore, we took advantage of the established observation that a fraction of EBV-infected B cells in the peripheral blood enter the lytic cycle when placed into tissue culture (18). Peripheral blood B cells were cultured at 37°C for 48 h at 0.5×10^6/ml in RPMI 1640 supplemented with 10% fetal calf serum. Acyclovir (Burroughs Welcome), an inhibitor of viral replication, was added to half of the culture at a final concentration of 0.1 mM. The cells were then analyzed by Gardella gel and PCR for the presence of linear viral genomes. The result is shown in Fig. 5. Abundant signals were detected in both the linear and episomal regions of the gel when the cells were incubated in the absence of acyclovir. To prove that this linear DNA was derived by viral replication, we compared the signal in the linear region to that obtained from the culture treated with acyclovir. As expected, the signal was dramatically reduced. The residual linear signal seen in the presence of acyclovir could have resulted from incomplete inhibition by the drug or nicking of episomes during cell death in the cultures. Noticeably, the signal in the episomal region was also reduced. Although the reason for this is unclear, it is likely to be a combination of loss of episomes in the acyclovir-treated cultures through shearing and the formation of new episomes through reinfection in the culture without acyclovir.

In conclusion, we have demonstrated that EBV persists in resting B cells in the peripheral blood as an episome. EBV is known to persist in proliferating cells as an episome, but this is the first demonstration that the virus also persists this way in a resting B-cell population. EBV infects resting B cells in vitro but does not circularize its genome until the cells become activated. Passage through the first G_1 appears to be required for the viral DNA to circularize, since inhibitors of RNA and protein synthesis block circularization (6). If the same process is required in vivo, then the resting EBV-infected cells in the peripheral blood must have been proliferating at some time in order to circularize their genomes.

We have previously proposed CD23⁻ B cells as a site of long-term EBV persistence (12). However, we did not demon-

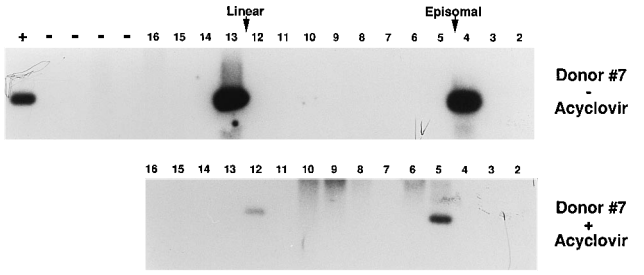

FIG. 5. Detection of linear genomes and viral reactivation in short-term cultures of peripheral blood B cells from normal donors. The experiment was performed as described in the legend to Fig. 3, except that the cells were incubated at 37°C for 48 h in the presence or absence of acyclovir prior to being loaded onto the Gardella gel. The samples from both cultures were analyzed in parallel under identical conditions. The PCR products from both samples were electrophoresed for Southern blotting on the same gel. +, positive control for the PCR of 60 Namalwa cells (two genome copies per cell); −, PCR analysis of slices from regions of the gel that should not contain EBV DNA, as a negative control. Gel slice numbers correspond approximately to the positions in Fig. 1. In all experiments, several no-DNA and negative-DNA (from the cell line BJAB) samples were analyzed simultaneously as negative controls (data not shown).

strate the presence of an intact viral genome in those cells nor did we show that the cells were latently infected. The observations made here address both of these issues by demonstrating the presence of an intact viral genome in the episomal, covalently closed conformation characteristic of latent infection. No linear genomes were detected.

Our conclusion is supported by previous studies using reverse transcription PCR that detected only latent transcripts in peripheral blood (2, 14, 17). However, those studies could not exclude the possibility that a significant fraction of the cells was undergoing lytic replication. For example, Chen et al. (2) and Tierney et al. (17) reported failing to detect mRNA for the Z transactivator that initiates the lytic cycle. However, a negative result is difficult to interpret. The Z transcript may be less stable or expressed at a lower level or for a shorter time in normal B cells than in cell lines. Even if cell lines are an accurate representation, the sensitivity of the published assays was approximately 1 in 10^5 for whole peripheral mononuclear cells (17) or 1 in 1.5×10^3 B cells (2). However, we have measured the frequency of EBV-carrying cells in peripheral blood of normal donors and it ranged from 1 in 2×10^5 to 10^7 whole mononuclear cells or 1 in 2×10^4 to 10^6 B cells. Therefore, the sensitivity of the reverse transcription PCR for Z is not high enough to exclude the possibility that a significant fraction of the cells are replicating the virus. Conversely, a positive result could not distinguish abortive reactivation from genuine replication. By comparison, we can readily detect a single cell that is replicating the virus. Since we know the frequency of EBV-infected B cells in our donors, we can calculate that fewer than 1 in 40 of the EBV-infected cells is replicating the virus.

The lack of viral replication in peripheral blood B cells can be explained by recent observations that the latent protein LMP-2 is the only viral protein consistently detected in these cells (2, 11b, 14, 17); LMP-2 is believed to block fortuitous signalling that could lead to viral reactivation (10). Although LMP-2 is a known cytotoxic T-lymphocyte target (9) it would not be detected because we have shown that the latently infected cells in peripheral blood are resting (11a) and lack the costimulatory molecule B7 (12) necessary to activate cytotoxic T lymphocytes.

The presence of LMP-2 is not consistent with previous models of EBV latency, based on Burkitt's lymphoma (8, 9), in which EBNA-1 only is expressed. LMP-2-restricted latency, to prevent fortuitous reactivation, in a B7-negative, resting B cell to escape cytotoxic T-lymphocyte recognition appears to be a better model for EBV persistence. If we are correct in believing that resting LMP-2-positive cells must have been proliferating at some point to circularize their viral genome, it is likely that this proliferating cell is the one that expresses the EBNA-1-only form of latency because it would allow the viral genome to be replicated in a latent form in an activated B7-positive B cell without being recognized and killed by cytotoxic T lymphocytes.

This is the first time that episomal DNA has been demonstrated for a normal tissue persistently infected with any human herpesvirus. "Endless DNA" has been demonstrated, by restriction enzyme digestion and Southern blotting for joined ends, for normal sites of latency with other herpesviruses (3). However, this type of analysis cannot distinguish single episomes from tandemly integrated genomes or large concatemeric complexes. The sensitivity of our approach means that it should be generally applicable for the demonstration of episomal DNA in rare cells latently infected with other herpesviruses and should allow distinction of whether cells are latently or lytically infected. Determining the relative contributions of lytic versus latent replication in herpesvirus-associated pathologies is of considerable importance in general because only the lytic form of replication is sensitive to the known antiherpesvirus drugs.

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